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ABSORPTION OF RIBONUCLEASE BY CELLS OF BONE MARROW AND EHRLICH'S ASCITES TUMORS

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Different authors have reported that living cells treated with ribonuclease (RNase) underwent important changes in growth and metabolism: eggs of amphibians (6,20) and sea urchins (16), oocytes of starfish (2), amoebas (3,4), Bacillus megaterium (13), onion root cells (1,14), tissue culture cells (8) and cancer cells (18,23).

The observed effects produced by RNase in different cells have led to the belief that the enzyme can penetrate through cell membranes. Whenever cells are not affected by the enzyme, it is assumed it did not penetrate into the cell.

We have already briefly reported (21) that RNase does not produce an effect - detectable by cytochemical or biochemical methods - in cells of bone marrow readily penetrated by RNase. Yet, RNase activity in treated cells was much higher than in untreated control cells.

In order to verify these facts and to explain them, if possible, we investigated the absorption in vitro of RNase by cells of bone marrow and Ehrlich's ascites tumors.

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MATERIALS AND METHODS

Ascites Tumor Cells - Cells of Ehrlich's ascites tumor were implanted in stock mice. After 6-14 days, the tumor cells were removed and used.

Bone Marrow Cells - Cells were removed from the femur and tibia of young rats, 1-3 months old, mixed in a homogenizer containing the following incubation medium: NaCl 0.85% 15 ml, glucose 5 ml(36 mg/ml in PO_4 buffer, M/10, pH 7.2). The cells were separated and suspended by centrifugation at 2000 rpm for 2 minutes.

Ribonucleic Acid (RNA) - The substrate used for testing RNase at different concentrations was prepared from fresh yeast by the method of Kay and Dounce (15). Experience showed that highly polymerized RNA was indispensible for obtaining accurate results with low concentrations of RNase. The substrate was dissolved in acetate buffer 0.2M, pH 5.0.

Ribonuclease (RNase) - RNase purchased from Armour Co. was used in these experiments. The RNase was labeled with ^{131}I, according to the method of Francis et al.(11). After seven consecutive dialyses (for 6 hours against a large amount of water) contamination was practically absent: chromatography and electrophoresis on paper indicated the radioactivity was uniquely localized in the protein. Specific radioactivity of RNase-^{131}I was about 1.5×10^6 counts/min/mg. The RNase-^{131}I was diluted 6 to 20-fold with fresh RNase (armour) in order to obtain the proper level of radioactivity.

Incubation of Cells in the Presence of RNase - Ascites tumor cells (in their normal plasma) and bone marrow cells (suspended in the glucose medium described above) were incubated in the presence of RNase-^{131}I under mechanical agitation at 37°C.

Extraction of RNase - After incubation, the cells were washed three times with a large volume of NaCl solution 0.9%. RNase-^{131}I was extracted with H_2SO_4 N/15 at 0°C for one hour. An aliquot was removed in order to determine the protein content of the entire suspension by the method of Lowry et al. (25).

The suspension in sulfuric acid was adjusted to pH 5.0 with dilute NaOH, centrifuged and the supernatant was tested for enzyme activity.

Measurement of RNase Activity - Incubation was at 25°C. Samples were tested after 2, 12 and 22 minutes. RNA not hydrolyzed by RNase was precipitated with a mixture of ethanol and glacial perchloric acid 10%. Precipitation was at 0°C for 45 minutes. The suspension was centrifuged; the supernatant was decanted and tested. Its UV spectrum was determined spectrophotometrically and the difference in absorption between 260 and 300 mμ was measured. The values were plotted as a function of the incubation temperature. The straight portion of the slope was used to measure RNase activity.

Measurement of Radioactivity - Cells, incubated with RNase-^{131}I, were washed three times with a large volume of NaCl solution 0.9%, suspended in water and deposited on glass planchets. These were dried in a stream of warm air and their radioactivity was measured with a Geiger counter. The glass planchets were dropped into a known volume of NaOH 0.1 N and the protein was determined by the method of Lowry et al. (25).

Autoradiography - A thin layer of cells, 6 μ thick, or sections were placed on agar layers prepared with Ilford G 5 agar, according to the method of Ficq and Gastovo (10) for sections about 100 μ thick (counts of ^{131}I are longer than those of ^{14}C).

RESULTS

1. Influence of the Concentration of Extracellular RNase

Bone Marrow Cells - In Figure 1, results are shown obtained by determining the enzymic activity of bone marrow cells after incubation with RNase, 2 or 10 mg/ml of suspended cells.

It may be seen the initial velocity of absorption was greater when the concentration of RNase was higher in the external medium. After 60 minutes of incubation, a maximum was attained and the initial RNase content of the cells was doubled. In Figure 2, results are shown obtained by measuring the radioactivity of cells incubated with RNase-^{131}I, 2 or 10 mg/ml of suspended cells. Similar results were obtained by both methods.

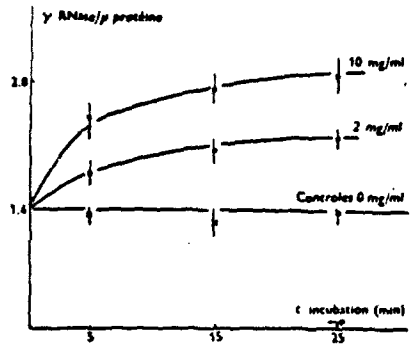

Fig. 1. Penetration of RNase into bone marrow cells incubated with RNase 2 or 10 mg/ml of suspended cells.

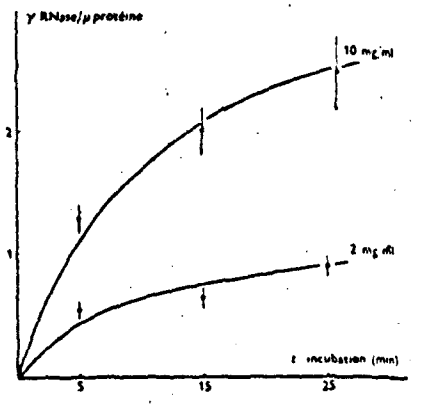

Fig. 2. Penetration of RNase-^{131}I into bone marrow cells incubated with RNase, 2 or 10 mg/ml of suspended cells.

Ascites Tumor Cells - In Figures 3 and 4 there are shown the effect of two concentrations of RNase, 2 and 10 mg/ml of suspended cells, or the

rate of enzyme absorption by the ascites tumor cells, removed 14 days after implantation. It may be seen, absorption was faster when the concentration of the enzyme was higher in the external medium.

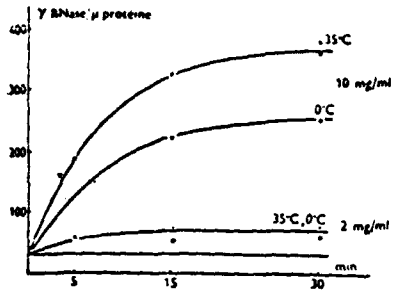

Fig. 3. Penetration of RNase into ascites tumor cells as a function of the concentration of RNase, 2 and 10 mg/ml of suspended cells, and as a function of incubation temperature 0°-35°C.

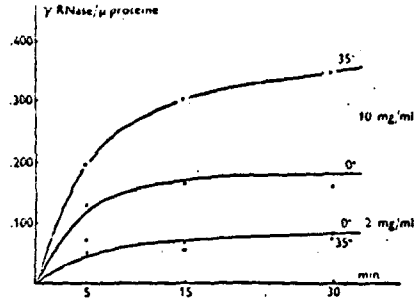

Fig. 4. Penetration of RNase-^{131}I into ascites tumor cells as a function of the concentration of the enzyme, 2 and 10 mg/ml of suspended cells, and as a function of incubation temperature 0°-35°C.

In Figure 5 there are shown the results of the absorption of RNase-^{131}I by ascites tumor cells removed from implanted tumors after 3 days (young cells, a), 14 days (middle-aged cells, b) and 21 days (old cells, c). It may be seen from the graph young cells absorbed RNase very rapidly and in large amounts, while in old cells relatively little enzyme was absorbed and the process was greatly slowed down. Also, one may note that after a very marked and very fast initial increase in radioactivity an abrupt decrease occurred and the values approached those obtained with the oldest cells.

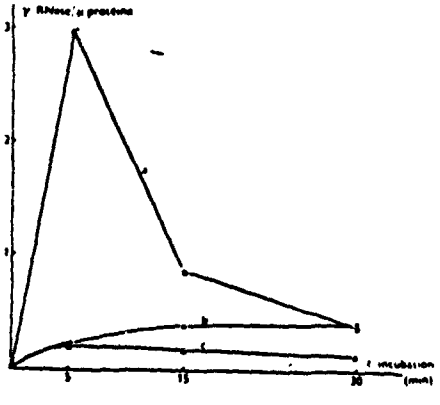

Fig. 5. Penetration of RNase-^{131}I (2mg/ml of suspended cells) into ascites tumor cells removed from tumors of different ages: a-cells removed from 3-day old tumors; b-14 day-old tumors; c - 21-day old tumors.

Examination of the autoradiographs obtained from the 6 μ thick layer of residue of ascites tumor cells or sections confirmed the results obtained by measuring radioactivity with a Geiger counter: young cells gave 0.41±0.07 counts/cell after 5 minutes and 0.24± 0.01 after 30 minutes of incubation.

2. Effect of Temperature on RNase Absorption

Bone Marrow Cells - When the enzymic activity of RNase in cells incubated between 0° and 37°C was measured, it was found the absorption of the enzyme was not influenced by the temperature.

Ascites Tumor Cells - Just as with bone marrow cells, ascites tumor cells absorbed RNase at the same rate at 0°C as they did at 37°C. As long as the RNase concentration in the external medium was greater by 8 mg/ml, more enzyme was absorbed at 37°C than at 0°C. This is shown in Figures 2,3 and 6. The autoradiographs of the cells incubated with RNase-^{131}I, 2 mg/ml of suspended cells, showed there was no difference between the cells incubated at 0°C (0.21 ± 0.01 counts/cell) and cells incubated at 37°C (0.22± 0.01 counts/cell).

3. Estimating the Surface Contamination of the Cells

The electrons emitted by ^{131}I produce very long counts near the ionization minimum (at least, at the point of particle emission). We were unable to localize with great precision the point of emission of the particles in the interior of the ascites tumor cells, which have an average diameter of 12μ, by means of nuclear emulsions. Therefore, we were unable to detect by autoradiography that portion of RNase that was bound to the cell membrane and that portion which penetrated into the cell. This is the reason why we decided to determine the amount of RNase absorbed on the cell surface by measuring the loss of enzymic activity of the suspension after washing the cells and separating cellular and cytoplasmic debris after crushing the cells in a sucrose solution.

Ascites tumor cells were incubated at 37°C for 20 minutes in the presence of RNase, 2 mg/ml of suspended cells. After incubation, the cells were washed with NaCl solution 0.9% under agitation. An aliquot was removed for the determination of RNase activity and the rest of the suspension was centrifuged. Cells were washed with fresh NaCl solution 0.9%. This operation was repeated six times. After the 7th washing, the remaining cells were crushed in a sucrose solution 0.25 M by means of a Teflon-coated piston homogenizer driven by a motor. The homogenate was centrifuged at 8000 g, the residue was again dissolved in sucrose solution and centrifuged at 6000 g.

Microscopic examination revealed the residue contained entire cells, not ruptured, and fragments of the cell membrane. The supernatant contained suspended cytoplasmic constituents. RNase activity of the residue was compared with a similar residue obtained at the start of the experiment, using

the same sample of cells, but not incubated with RNase.

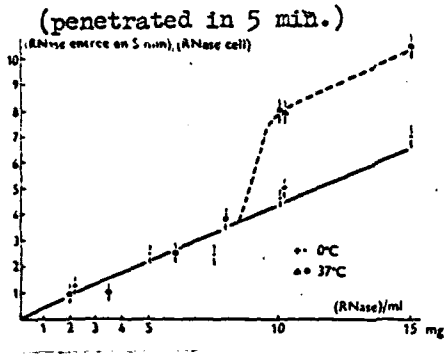

Fig. 6. Effect of RNase concentration in the incubation medium on the amount of enzyme which penetrated the cell after 5 minutes between 0° and 37°C.

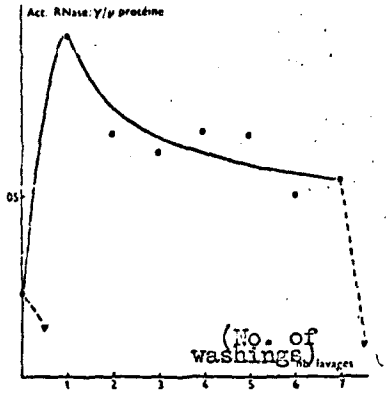

Fig. 7. Effect of the number of washings on RNase activity of ascites tumor cells incubated with RNase. • enzymic activity of cells before incubation; o-enzymic activity of cells incubated for 20 minutes at 37°C with RNase 2 mg/ml of suspended cells; △ enzymic activity of membranes obtained from centrifuged homogenized cells.

It may be seen in Figure 7 that two to three washings sufficed to eliminate RNase loosely bound to the cells. Also, it may be seen the cellular debris were not richer in RNase than the cells not treated with the enzyme: the most important RNase activity occurred in the cytoplasm of the cells.

DISCUSSION OF RESULTS

Penetration of the Enzyme into Cells - Our results showed that RNase (mol. wt. 13,000) was capable of passing through cell membranes and of penetrating into cells of bone marrow and ascites tumors. In fact, the measurement of RNase activity in membranes and in the cytoplasm of disrupted cells indicated that the activity was the same whether or not the cells were incubated with RNase. However, in the latter case, RNase activity was three times stronger in the cytoplasm.

Mechanism of Penetration - The results which we obtained from studying the effect of temperature on the penetration of RNase into cells confirmed, again, the observations made by Brachet et al. (5) and Schumaker (27) with amoebas and those made by Easty et al. (9) with ascites tumor cells. The first mentioned group of authors demonstrated that the temperature did not affect the rate of penetration of the enzyme, while the second group observed, on the contrary, that the rate was more rapid at 38.5°C than

at 18°C. Our results indicated that the temperature had no effect on the penetration of RNase as long as its concentration in the medium was sufficiently high. Obviously, the mechanism of penetration of RNase is complex: it is possible that RNase in low concentrations penetrates simply by diffusion, this process being influenced by the temperature, while at higher concentrations penetration occurs because of cell membrane activity through pinocytosis. This process was described by Lewis (24), Mast and Doyle (26), Holter and Marshall (12) in tissue cultures and amoebas, and it had been observed in ascites tumors by Easty et al (9). It is probable that the membrane transport is more rapid when the enzyme is more concentrated.

The quantity of RNase which penetrated into ascites tumor cells varied strongly with the age of the tumor. This supports the notion that several mechanisms are involved in order to assure the penetration of the enzyme. Cells removed from a recently implanted tumor (1-3 days old) absorbed, in some instances, great amounts of RNase which was rapidly returned to the medium, while cells from a 7-12 day-old tumor absorbed RNase slower, but more steadily.

When the results are compared with those obtained by Chapman-Andresen and Prescott (7) with amoebas, it must be admitted that RNase penetrated only by pinocytosis into young cells and by diffusion into old cells.

Effects of the Concentration of the Enzyme on Cellular Metabolism - Our experiments furnished an explanation of data reported earlier by one of us (L.L., 17) which showed that the effect of RNase on the metabolism of ascites tumor cells depended directly on the age of the suspension used: in young suspensions, the extracellular RNase produced a drop in RNA content and very rapid lysis of the young cells. In suspensions of older cells, an important synthesis of RNA was noted and the cellular lysis was different.

Depending on the level of intracellular concentration of RNase attained, the effects produced by the enzyme were different. When the initial content was not more than double there was no effect or only a small effect (aged cells). On the contrary, when the content was increased 2-30-fold, RNA synthesis occurred (as observed in middle-aged ascites tumor cells). Finally, when the cells absorbed too much RNase, the RNA content fell rapidly and the cells lyzed (especially young cells).

RNase added to the medium produced effects which depended simultaneously on the concentration of the enzyme in the medium and on its initial concentration within the cell. It was noted that a quantity of added RNase produced a much greater effect when the cells subjected to its action had a below normal RNase content. Also, we know (19,22) that cancer cells, in general, have a much lower RNase content than analogous normal cells.

This phenomenon might very well be caused by the selective action of RNase on tissues of an animal carrying a solid tumor: it was observed that only the cancerous tissues were penetrated by RNase.

SUMMARY

The absorption of RNase by ascites tumor cells and bone marrow cells was followed by measuring enzymic activity and the radioactivity of cell suspensions incubated in the presence of enzyme labeled with ^{131}I.

Our experiments indicated that the enzyme penetrated into the interior of the cells and that the rate of penetration depended on the concentration of RNase in the medium.

The penetration of the enzyme into ascites tumor cells was not affected by temperature as long as the concentration of RNase in the external medium did not exceed a certain threshold value. These facts suggested that several mechanisms-diffusion, membrane activity, pinocytosis-- all differentially influenced by temperature, facilitate the penetration of RNase into the cell.

In the light of these new results, the effects of RNase on the metabolism of normal and cancerous cells were discussed.

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